Remarks

Claims 20-23, 35, 36, and 38 were the subject of the office action dated April 14, 2008. These claims are again presented for further consideration.

Claim 38 is amended so that the terminology has even more clear basis. For example, paragraph 31 of the published application (US 2004-0254364) states "Polynucleotides encoding any known Bt toxins or those yet to be discovered and active fragments thereof (see, for example, U.S. Pat. No. 5,710,020) can be used in accord with the teachings herein [emphasis added]." For purposes of even greater clarity, "active fragment" is noted in the plain language of claim 38 to mean an insecticidal fragment.

Claims 20-22, 35, and 38 remain rejected under 35 USC §102(b) as being anticipated by Marzari. The applicants respectfully traverse this rejection.

Claim 20 now further clarifies that the Cry toxin is insecticidal. Marzari clearly does not teach an insecticidal Cry toxin that is displayed on the surface of the phage.

Without Domains I, II and III of a *Bacillus thuringiensis (B.t.) Cry* toxin, there is no insecticidal activity, and the "toxin" is not "active." Anyone skilled in the art of working with Bt knows that "active" toxins are toxins that are insecticidal.

As stated in the first paragraph of the Background section of the subject application, "These studies revealed that the activated form (amino acid residues 33-609 of the Cry1Aa protoxin) of both of these polypeptides consists of three globular domains. This tertiary structure, as well as amino acid homologies and secondary structures within the domains led to assignment of putative functions for each."

Dr. Adang explained in his prior declaration, "In contrast [to Marzari], the insecticidal activity of the core toxin that is displayed according to our invention is a key component. For example, with the fragments described by Marzari, one could screen with the binding fragment for binding, but one could not then use the fragment to screen for insecticidal activity."

To further illustrate this, attached is Hofte (1989). See in particular Figure 1, which shows that the "core toxin" comprises all five "blocks" – corresponding to the first 600-700 amino acids or so. (These five blocks are included in the three domains discussed herein.) Anything less than this is NOT ACTIVE / NOT INSECTICIDAL.

As illustrated by Figure 1 of Marzari, only one construct of Marzari ("BtL") had all three domains required for insecticidal activity. "BtS" was a shorter construct, comprising part of domain I and half of domain II (without domain III). "BtDII" had only domain II. "Btl2" had only loop 2 of domain II.

Page 30 of Marzari is quoted on the first page of Dr. Adang's declaration signed on November 26, 2007. Again, Marazari states there:

> Cloning of a large fragment [BtL] corresponding to the activated [core] toxin caused slowed bacterial growth, but did not cause lysis unless the bacteria were also infected with a helper phage. We feel that this may arise from the insertion of a functional toxin pore molecule into the cell membrane following phage extrusion. The induction of toxicity is probably responsible for the lack of display observed. [emphasis added]

Clearly, Marazari does not anticipate what is now claimed – a properly displayed, insecticidal Cry protein.

In light of the foregoing, the withdrawal of this rejection is respectfully requested.

Claims 20-22, 35, 36, and 38 remain rejected as being obvious in light of Marazari, Stewart, and Masson. The applicants respectfully traverse this rejection.

Marzari is the primary reference. As discussed in the applicants' previous response and in Dr. Adang's declaration, Marzari teaches away from using insecticidal Cry proteins. Marzari teaches the use of subdomains / non-insecticidal fragments of Cry proteins. The non-insecticidal fragments taught by Marzari were thought to be responsible for binding of Bt Crys to insect cellular receptors. Thus, Marzari was screening for binding potential. Because of cellular toxicity and lack of display as discussed above, Marzari taught against the use of full, insecticidal Cry toxins.

In addition, as Marzari taught that full, insecticidal constructs would not be properly displayed for screening, the functional phage fusions now claimed clearly could not have been predicted in light of Marzari.

To address concerns set forth in the middle of page 10 of the office action, the Background section of the subject application, Dr. Adang's prior declaration, and the attached Hofte 1989 reference all provide evidence (actually, they clearly show and explain) that the Marzari (non-insecticidal) fragments that arguably were properly displayed by Marzari were

non-toxic. The structural differences between the non-toxic fragments of Marzari are discussed in those three sources, above in this response, and in the applicants' prior response.

The statement in the office action bridging pages 10-11 of the office action (that loop 2 of domain II would be considered toxic) is simply completely contrary to what is well-known in the Bt art. The examiner was very observant to note that this loop (loop 2) of domain II could be involved with binding. However, mere binding of a larger toxin to a receptor is clearly only one component of a larger, overall mechanism of toxicity. In order to be toxic to an insect, the toxin must have all three domains at a minimum, as discussed in more detail above.

Marzari's phage might contain a non-toxic fragment wherein the fragment is structurally similar to a fragment of an insecticidal Cry protein. However, in no way could Marzari be said to teach or suggest the successful display of insecticidal Cry proteins. Insecticidal Cry proteins are much different from (non-insecticidal) sub-domains thereof.

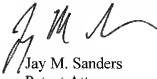
The applicants also wish to note that many of the passages of Marzari that are cited in the office action clearly refer to "regions" or "fragments" of an insecticidal Cry protein. Again, sub-domains with a function for activity of a larger protein (whether the sub-domains are involved with membrane insertion, pore formation, receptor binding, or the like) are much different from insecticidal proteins comprising all the sub-domains required for insecticidal activity. Otherwise, it would be like arguing that a car engine, car tires, and car axles are the same as a car, and that each could be driven independently of the other components. The term "insecticidal" should re-emphasize that the term "active" was well-known in the Bt art, as discussed above.

In light of all the foregoing, the applicants respectfully request the withdrawal of this rejection for obviousness.

The applicants believe that the subject application is in condition for allowance, and such action is respectfully requested. The applicants invite the Examiner to call the undersigned if clarification is needed on any of this response, or if the Examiner believes a telephonic interview would expedite the prosecution of the subject application to completion.

The Assistant Commissioner is hereby authorized to charge any fees under 37 CFR §§1.16 and 1.17 as required by this paper to Deposit Account 19-0065.

Respectfully submitted,



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JMS/mrc
Attachments: Petition and Fee for Extension of Time Under 37 CFR §1.136(a)
Hofte (1989)

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Insecticidal Crystal Proteins of *Bacillus thuringiensis*

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INTRODUCTION

Bacillus thuringiensis is a gram-positive soil bacterium characterized by its ability to produce crystalline inclusions during sporulation. These inclusions consist of proteins exhibiting a highly specific insecticidal activity (reviewed in references 4 and 97). Many *B. thuringiensis* strains with different insect host spectra have been identified (9). They are classified into different serotypes or subspecies based on their flagellar antigens. Most strains are active against larvae of certain members of the Lepidoptera, but some show toxicity against dipteran (reviewed in reference 22) or coleopteran (53) species. For several crystal-producing strains, no toxic activity has yet been demonstrated.

B. thuringiensis crystalline inclusions dissolve in the larval midgut, releasing one or more insecticidal crystal proteins (also called δ-endotoxins) of 27 to 140 kilodaltons (kDa). As described in the following section, most crystal proteins are protoxins that are proteolytically converted into smaller toxic polypeptides in the insect midgut. The activated toxin interacts with the midgut epithelium cells of susceptible insects. Electrophysiological (32) and biochemical (49) evidence suggests that the toxins generate pores in the cell membrane, thus disturbing the osmotic balance. Consequently, the cells swell and lyse. The larva stops feeding and eventually dies. For several *B. thuringiensis* toxins, specific high-affinity binding sites have been demonstrated to exist on the midgut epithelium of susceptible insects (37, 38). This could, at least in part, explain the extreme specificity of these proteins.

Formulations of *B. thuringiensis* have been used for more than two decades as biological insecticides to control agricultural pests and, more recently, insect vectors of a variety of human and animal diseases. Recently, the cloning of insecticidal crystal protein genes (97) and their expression in plant-associated microorganisms (72) or transgenic plants (5, 23, 90) has provided potentially powerful alternative strategies for the protection of crops against insect damage.

These applied aspects are to a large extent responsible for an increased interest in this bacterium and its crystal proteins in recent years. Extensive screening programs are being carried out by various groups to search for *B. thuringiensis* strains with new insecticidal spectra. Numerous publications report the identification of crystal proteins and the cloning and sequencing of crystal protein genes. One problem related to this is the lack of a uniform nomenclature for these genes and their products, which makes the literature rather confusing.

In this review, we will present an update of the current knowledge of *B. thuringiensis* crystal proteins and their genes. We will also propose a nomenclature and classification scheme for crystal proteins based on their structure (deduced from the deoxyribonucleic acid [DNA] sequence) as well as their host range.

DIVERSITY AND CLASSIFICATION OF CRYSTAL PROTEIN GENES

To date, nucleotide sequences have been reported for 42 *B. thuringiensis* crystal protein genes. Several sequences are identical or nearly identical and thus represent the same gene or slight variants of the same gene. Taking this into account, 14 distinct crystal protein genes remain. Several lines of evidence, summarized below, suggest that 13 of these genes—the so-called *cry* (crystal protein) genes—specify a family of related insecticidal proteins (Cry proteins). These 13 genes have been divided into a minimum number of major classes (four) and several subclasses characterized by both the structural similarities and the insecticidal spectra of the encoded proteins. The four major classes are Lepidoptera-specific (I), Lepidoptera- and Diptera-specific (II), Coleoptera-specific (III), and Diptera-specific (IV) genes. Additional classes could be proposed and may be added later, for example, to include genes coding for nontoxic crystal

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TABLE 1. Insecticidal crystal protein genes of *B. thuringiensis*[a]

Gene type	Host range[b]	No. of amino acids	Predicted mol mass (kDa)	Other gene designations	Reference (holotype)[c]
cryIA(a)	L	1,176	133.2	4.5-kb gene (56), *cryI-1* (85)	79
cryIA(b)	L	1,155	131.0	5.3-kb gene (56), *kurhd1* (27), *bt2* (39), *cryI-2* (85)	92
cryIA(c)	L	1,178	133.3	6.6-kb gene (56)	3
cryIB	L	1,207	138.0	*cryA4* (7), type B (41)	7
cryIC	L	1,189	134.8	Type C (41), BTVI (42), *Bta* (77)	42
cryID	L	1,165	132.5		Höfte, unpublished
cryIIA	L/D	633	70.9	P2 gene (17), *cryB1* (98)	17
cryIIB	L	633	70.8	*cryB2* (98)	98
cryIIIA	C	644	73.1	*cryC* (18)	35
cryIVA	D	1,180	134.4	130-kDa endotoxin gene (96), 125-kDa protein gene (6), ISRH3 (81)	96
cryIVB	D	1,136	127.8	130-kDa endotoxin gene (106), ISRH4 (82), *Bi8* (13), 135-kDa protein gene (6)	13
cryIVC	D	675	77.8	ORF1 (88)	88
cryIVD	D	643	72.4	*cryD* (16)	16
cytA	D/cytol.	248	27.4	27-kDa toxin gene (91)	91

[a] See text for description and gene designations; designations approved by the Nomenclature Committee (D. J. Nierlich, Chairman) of the Publications Board of the American Society for Microbiology.

[b] Specified host ranges: L, Lepidoptera; D, Diptera; C, Coleoptera; cytol., cytolytic and hemolytic.

[c] Reference of the first submitted publication describing the gene type. The number of amino acids and predicted molecular masses are derived from the holotype sequences.

proteins. However, the relationship of such genes to those described below is not known, since the DNA sequences of genes coding for nontoxic proteins have not yet been determined. One crystal protein gene of *B. thuringiensis* subsp. *israelensis* codes for a 27-kDa protein that exhibits cytolytic activity against a variety of invertebrate and vertebrate cells, and this gene is totally unrelated structurally to the *cry* genes. On this basis, we propose a separate designation for the gene coding for the 27-kDa protein: *cytA* for cytolytic crystal protein. Table 1 lists the genes assigned to the four major *cry* classes and to the *cyt* class.

cryI Genes

The Lepidoptera-specific crystal proteins are undoubtedly the best-studied crystal proteins. The 20 *cryI* sequences that have been reported are listed in Table 2. Six different genes (Table 1) can be recognized among the 20 sequences. All 20 genes encode 130- to 140-kDa proteins, which accumulate in bipyramidal crystalline inclusions during the sporulation of *B. thuringiensis*. As stated above, these proteins are protoxins which solubilize in the alkaline environment of the insect midgut and are proteolytically converted by crystal-associated or larval-midgut proteases into a toxic core fragment of 60 to 70 kDa. This activation can also be carried out in vitro with a variety of proteases (reviewed in reference 97).

The toxic domain is localized in the N-terminal half of the protoxin. This was demonstrated for the CryIA(b) (39) and CryIC (H. Höfte, unpublished data) proteins through N-terminal amino acid sequencing of the trypsin-activated toxin. Both proteins are cleaved at a homologous position in the sequence (residues 29 and 28, respectively). Nagamatsu et al. (71) determined the N-terminal amino acid sequence of the tryptic core fragment of a *B. thuringiensis* subsp. *dendrolimus* crystal protein. This sequence corresponds to the CryIA sequence starting from residue 29. The proteolytic cleavage site is highly conserved for the other CryIA and the CryID proteins as well, suggesting that for these proteins, the N terminus of the toxic fragment is localized at the same position. CryIB, however, is very different from the other CryI proteins in this region. It is not known whether this protein is also processed at the N terminus. Deletion analysis of several *cryI* genes [*cryIA(a)* (78), *cryIA(b)* (39, 92), *cryIA(c)* (3), and *cryIC* (77)] further confirmed that the 3′ half of the protoxin is not absolutely required for toxic activity. The shortest reported toxic fragment was localized between codons 29 and 607 for CryIA(b) (39). Further removal of 4 codons from the 3′ end (92) or 8 codons from the 5′ end completely abolished the toxic activity of the gene product. Similar observations were made for the *cryIA(a)* (78) and *cryIA(c)* (3) genes.

The *cryI* genes can be distinguished from the other *cry* genes simply by sequence homology (>50% amino acid identity [Table 3]). Three of these genes, *cryIA(a)*, *cryIA(b)*, and *cryIA(c)*, show more than 80% amino acid identity and have therefore been considered as a separate subgroup. These three genes were previously designated as the 4.5-, 5.3-, and 6.6-kilobase (kb) genes, respectively, on the basis of the size of the *Hin*dIII restriction fragment containing the 5′ end of the genes (56). The nucleotide differences between these three genes are localized mainly in a limited section of the region encoding the toxic fragment (97). The recently identified *cryIB*, *cryIC*, and *cryID* genes differ much more from each other and from the *cryIA* genes (Fig. 1A).

A crystal protein gene from *B. thuringiensis* subsp. *aizawai* IC-1 is listed in Table 2 as a *cryIA(b)* gene, despite the fact that the protein has dual host specificity. As described in a later section on specificity, the toxicity of the IC-1 protein to lepidopteran or to dipteran insects depends on the source of the proteolytic enzyme that generates the toxin (29, 31). The decision to include the IC-1 gene in the *cryIA(b)* subclass (rather than creating a new class) was based on the structure of the gene product: the amino acid sequence differs from the holotype by only four amino acids.

Interestingly, comparison of the predicted amino acid sequences shows that the C-terminal half is highly conserved for all *cryI* genes (Fig. 1A). It is unclear whether the sequence conservation in this region reflects any functional significance. As shown above, this domain does not seem to

TABLE 2. Overview of reported crystal protein gene sequences

Crystal protein gene	*B. thuringiensis* subsp. and/or strain	No. of amino acid differences from holotype sequence[a]		Reference
		Protoxin	Toxin[b]	
cryIA(a)	*kurstaki* HD-1	H	H	79
	aizawai	3	2	85
	kurstaki HD-1	1	0	52
	sotto	24	3	83, 84
	entomocidus	1	0	64a
cryIA(b)	*berliner* 1715	H	H	2, 92
	berliner 1715	2	0	39
	kurstaki HD-1	2	2	52
	kurstaki HD-1	5	4	27, 88[c]
	aizawai 1PL-7	4	2	73
	kurstaki HD-1	6	2	23
	kurstaki NRD-12	10	6	33
	aizawai IC-1	4	4	30
cryIA(c)	*kurstaki* HD-73	H	H	3
cryIB	*thuringiensis* HD-2	H	H	7
	entomocidus HD-110	1	1	Höfte, unpublished
cryIC	*entomocidus* 601	H	H	42
	aizawai HD-137	7[d]	7	77
	entomocidus HD-110	2	2	Höfte, unpublished
cryID	*aizawai* HD-68	H	H	Höfte, unpublished
cryIIA	*kurstaki* HD-263	H		17
	kurstaki HD-1	0	0	98
cryIIB	*kurstaki* HD-1	H		98
cryIIIA	*san diego*	H		35
	tenebrionis	0	0	40, 69, 81
	EG2158	0	0	18
cryIVA	*israelensis*	H	H	96
	israelensis	4	1	82
cryIVB	*israelensis*	H	H	13
	israelensis	1	1	89
	israelensis	3	3	82
	israelensis	97	78	106
cryIVC	*israelensis*	H	H	88
cryIVD	*israelensis*	H		16
cytA	*israelensis*	H		91
	morrisoni PG-14	1	1	19
	israelensis	0	0	93
	morrisoni PG-14	1	1	24

[a] The first reported sequence of a gene type is considered the holotype (H) sequence. Subsequently reported amino acid sequences are defined by the number of amino acid changes with respect to the holotype sequence.

[b] Toxin. The N-terminal half of the crystal protein, delineated by the C-terminal amino acid of the conserved amino acid sequence block 5 (Fig. 2).

[c] Corrections to the sequence in reference 88 (T. J. Pollock, personal communication) show that it is identical to that in reference 27.

[d] The reported sequence represents the first 823 residues (481.

play a role in toxicity. However, it is interesting that almost all cysteine residues are localized in the C-terminal half and that disulfide bonds have been implicated in the maintenance and the unusual solubility properties of the crystals (63). Hence, the C-terminal half may be intimately involved in crystal formation, and the conservation of sequences in this part of the molecule is apparently sufficient to allow the coassembly of different crystal proteins in the same crystals.

Distribution of CryI proteins among different *B. thuringiensis* strains. Traditionally, the protein composition of *B. thuringiensis* crystals has been studied through polyacrylamide gel electrophoresis (10, 46) or immunologically by using polyclonal antisera against purified crystals (57). These methods showed that some crystals contained more than one protein. A genetic approach became possible with the cloning of crystal protein genes. The use of gene-specific probes led to the discovery that various subspecies of *B. thuringiensis* contained one, two, or three closely related genes (54, 56). These three genes were found in a number of different strains or subspecies (e.g., *B. thuringiensis* subsp. *kurstaki* HD-1 contains all three genes, strain HD-1 Dipel has the *cryIA(a)* and *cryIA(c)* genes, *B. thuringiensis* subsp. *thuringiensis* HD-2 contains *cryIA(b)* and *cryIA(c)*, etc.).

Recently, an alternative approach was described in which monoclonal antibodies were used to identify single proteins in crystal preparations. Huber-Lukac et al. (43) described 10 monoclonal antibodies generated against purified crystals from *B. thuringiensis* subsp. *kurstaki*. Investigation of the crystals of 14 *B. thuringiensis* strains with these antibodies revealed clear-cut differences in immunoreactivity. However, in this study, no direct correlation was made between the reactivity with certain monoclonal antibodies and the presence of certain crystal protein types.

In another study, monoclonal antibodies were used to distinguish the CryIA, CryIB, and CryIC proteins in crystal preparations (41). Table 4 shows the results of a survey of 29 strains of 11 serotypes by using 35 monoclonal antibodies. CryIA is the most common crystal protein type and was present in all but one strain tested; CryIB and CryIC were less common. The former occurred in five subspecies, whereas CryIC was present only in *B. thuringiensis* subspp. *aizawai* and *entomocidus*. Certain strains of *B. thuringiensis* subspp. *galleriae* and *morrisoni* contained proteins that reacted only with a limited subset of the antibodies specifying a crystal protein type. It will be interesting to see to what extent these crystal proteins differ from the already-known crystal protein types with regard to structure and insect specificity. It should be noted that the monoclonal antibodies used in the above studies may not have detected all of the toxins in the crystals. Similarly, subtle variations in amino acid sequence which might be responsible for differences in host spectrum might have escaped detection. It is clear, however, that monoclonal antibodies can significantly speed up the identification of crystal proteins produced by newly isolated *B. thuringiensis* strains and are therefore a powerful tool in screening for strains with new insecticidal properties. They allow the rapid identification not only of known crystal proteins but, more importantly, also of crystal proteins with as yet unknown structural characteristics (e.g., as was shown for strains of *B. thuringiensis* subspp. *galleriae* and *morrisoni*). In this way, strains producing crystal proteins with unique structural properties—and possibly also unique insecticidal spectra—can be preselected prior to the more time-consuming bioassays.

In toto, these data clearly demonstrated that many strains produce several crystal proteins simultaneously and that the

TABLE 3. Percent amino acid identity between *cry*-encoded proteins

Crystal protein	% Amino acid identity[a] for:								
	CryIA(b)	CryIA(c)	CryIB	CryIC	CryID	CryIIIA[b]	CryIVA	CryIVB	CryIVC[b]
CryIA(a)	90	82	55	67	71	25	27	27	22
CryIA(b)		86	56	66	71	28	27	27	23
CryIA(c)			55	67	70	23	28	24	24
CryIB				58	56	34	26	28	22
CryIC					70	31	26	30	21
CryID						23	28	28	22
CryIIIA							23	19	26
CryIVA								54	29
CryIVB									29

[a] For the calculation of this percentage, pairs of amino acid sequences were aligned by using the GENALIGN program (IntelliGenetics), with a gap penalty of zero. The number of matched amino acids was divided by the number of residues of the longest sequence of the two, using the entire deduced sequence. CryII and CryIVD are not included, because there is little or no significant amino acid identity between these two proteins and the others shown in the table. Because 100% matches are not indicated, CryIA(a) has been omitted from the columns and CryIVC has been omitted from the rows.

[b] The comparison was made by using only the putative toxin fragments (residue 1 to the C-terminal residue of conserved sequence block 5 of Fig. 2).

same (or very similar) crystal proteins occur in *B. thuringiensis* strains of different subspecies. This mobility of crystal protein genes among strains of *B. thuringiensis* subspecies is not unexpected, since most genes are localized on large conjugative plasmids (reviewed in reference 11). In addition, the observed association of several *cryIA* genes (55, 61) and *cryIVB* (6) (see below) with IS elements (64) and/or transposonlike structures could contribute to their mobility.

The insecticidal activity of *B. thuringiensis* crystal proteins has traditionally been investigated by using crude preparations of spore-crystal mixtures (9). The results of these studies, however, are difficult to interpret. Spore-crystal preparations generally also contain other toxic agents, such as the beta-exotoxin (58) and toxic spore components. In addition, in some cases it is not known whether the germination of spores inside the larvae contributes to the observed toxic effects. Hence, these earlier studies did not yield conclusive data that toxicity was due solely to the activity of the crystal proteins. In some recent investigations, gradient-purified crystals were used in toxicity assays (45, 60). However, since many *B. thuringiensis* strains simultaneously produce more than one crystal protein, it is still difficult to accurately determine the toxicity spectrum of individual proteins from these studies.

Preparations containing single crystal proteins have been obtained in the following ways: through protein purification (105), through the introduction (by cloning or conjugation) and expression of the corresponding genes in heterologous hosts (see the references cited in Table 2), or by using *B. thuringiensis* strains that produce only one crystal protein (41). The toxicity data for individual *cryI*-specified proteins obtained so far are summarized in Table 5. The proteins, purified from *B. thuringiensis* crystals consisting of a single protein [CryIA(c) and CryIB] or from recombinant *Escherichia coli* clones, were solubilized in an alkaline buffer (pH 10) with reducing agents prior to the bioassay. These data clearly demonstrate that single crystal proteins are highly specific for certain lepidopteran species. For instance, CryIB and CryID were active against only one of the lepidopteran

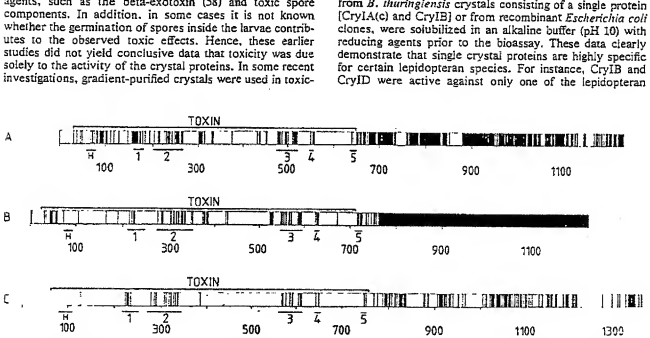

FIG. 1. Amino acid sequence comparison of *B. thuringiensis* crystal proteins. Sequences were aligned by using the GENALIGN program (IntelliGenetics). Gaps that were added for optimal alignments are not indicated. Vertical lines represent amino acids that are conserved for all lepidopteran crystal proteins (CryI) (A); all dipteran crystal proteins (CryIV) (B); and all CryI, CryIII, and CryIV crystal proteins (except CryIVD) (C). The positions of the five conserved sequence blocks (see text and Fig. 2) are underlined. The CryII proteins show significant homology only to the sequence of block 1. Vertical lines in the C-terminal half (outside the toxin-encoding region) represent amino acids conserved for the CryIVA and CryIVB proteins in panel B and for all crystal proteins specified by *cry* genes (except CryII, CryIII, and CryIVC and CryIVD) in panel C. Abbreviation: H, hydrophobic transmembrane sequence (20) present in all crystal proteins except CryII and CryIVD. Numbers refer to positions in the sequence after alignment (amino acids plus gaps).

TABLE 4. Distribution of three crystal protein types among different *B. thuringiensis* strains as determined by reaction with monoclonal antibodies[a]

Flagellar serotype	*B. thuringiensis* subsp.	Strain	Presence of following protein[b]:		
			CryIA[c]	CryIB	CryIC
1	*thuringiensis*	HD-2, Berliner 1715	+	+	–
		HD-14	+(a)	–	–
		4412	+	+	–
3a	*alesti*	HD-4	+	–	–
3a3b	*kurstaki*	HD-1	+	–	–
		HD-73	+ (c)	–	–
4a4b	*dendrolimus*	HD-7	+	–	–
		HD-37	+ (a)	–	–
4a4c	*kenyae*	HD-5, HD-64, HD-136	+ (c)	–	–
		HD-551	+	+	–
5a5b	*galleriae*	HD-8, HD-129	+	?	?
6	*entomocidus*	HD-110, 4448	+ (a)	+	+
6	*subtoxicus*	HD-10	+	+	–
7	*aizawai*	HD-11, HD-68	+	–	–
		HD-127, HD-854, HD-229, HD-133, HD-137	+	–	+
		HD-272	+	+	–
		HD-o47	+	–	+
8a8b	*morrisoni*	HD-12	+	?	–
9	*tolworthi*	HD-121	+	–	–

[a] See reference 41.

[b] Proteins that react with only some of the monoclonal antibodies that specify a crystal protein type are designated by ?. All CryIA-specific antibodies recognized CryIA(b); two did not bind to CryIA(a); two other monoclonal antibodies did not react with CryIA(c). Hence, the last two crystal proteins could be distinguished, provided that no other CryIA proteins were present in the crystals.

[c] The CryIA subtype (a or c) is indicated when known.

species tested. An interesting observation is that the three CryIA proteins, which are structurally very closely related, also show largely overlapping activity spectra.

cryII Genes

The *cryII* genes encode 65-kDa proteins which form cuboidal inclusions in strains of several subspecies: *B. thuringiensis* subsp. *kurstaki* HD-1 and 14 other strains, *B. thuringiensis* subsp. *thuringiensis* Berliner, and *B. thuringiensis* subspp. *tolworthi* and *kenyae* (103, 104; W. R. Widner, unpublished results). These crystal proteins were previously designated as P2 proteins, as opposed to the 130-kDa P1 crystal proteins present in the same strains (105).

The first *cryIIA* gene was cloned from *B. thuringiensis* subsp. *kurstaki* HD-263 and expressed in *Bacillus megaterium* (17). Cells producing the CryIIA protein were toxic for the lepidopteran species *Heliothis virescens* and *Lymantria dispar* as well as for larvae of the dipteran *Aedes aegypti*. Widner and Whiteley (98) cloned two related genes (*cryIIA* and *cryIIB*) from *B. thuringiensis* subsp. *kurstaki* HD-1. Both genes encode proteins of 633 amino acids with a predicted molecular mass of 71 kDa, slightly larger than the apparent molecular mass determined for the P2 proteins produced in *B. thuringiensis*. Both genes were expressed in *E. coli*, and the recombinant proteins were purified. Although the two proteins are highly homologous (87% amino acid identity), they differ in their insecticidal spectra. CryIIA is active against both a lepidopteran (*Manduca sexta*) and a dipteran (*A. aegypti*) species, whereas *cryIIB* is toxic only to the lepidopteran insect. The decision to classify the protein encoded by the latter gene as CryII (rather than CryI) is based on the structural similarity of CryIIA and CryIIB and on the fact that the CryII proteins show a rather limited homology to the other Cry proteins (see the section on conserved sequences). The DNA sequence further indicated that *cryIIA* is the distal gene of an operon, consisting of three open reading frames (*orf1*, *orf2*, and *cryIIA*). The gene products of *orf2* and *cryIIA* could be detected in cuboidal crystals in several *B. thuringiensis* subspecies. It is unclear whether the *orf1* and *cryIIB* products are also present in the crystals. The *orf2* gene product, which is highly immunogenic, has an unusual repeated structure; the functions of the proteins encoded by *orf1* and *orf2* are not known.

The reported sequences of the two previously described *cryIIA* genes (17, 98) are identical, except that Donovan et al. (17) identified an open reading frame of 590 rather than 633 amino acids. This difference is the result of a sequencing error (the insertion of one additional thymidine residue [W. P. Donovan, personal communication]). As shown below, the *cryII* genes show a rather limited homology to the other *cry* genes.

cryIII Genes

Three Coleoptera-specific *B. thuringiensis* strains have been described so far: *B. thuringiensis* subsp. *tenebrionis* (53), *B. thuringiensis* subsp. *san diego* (36), and *B. thuringiensis* EG2158 (18). Each of the strains produces rhomboidal crystals containing one major protein. Cloning and sequencing demonstrated the presence of the same crystal protein gene in all three strains. The gene, expressed in *E. coli*, directs the synthesis of a 72-kDa protein toxic for the Colorado potato beetle (*Leptinosarsa decemlineata*). This protein is converted into a 66-kDa toxin by spore-associated

TABLE 5. Toxicity of crystal proteins against five Lepidoptera species[a]

Cry protein	*B. thuringiensis* subsp. and strain	LC_{50} of protein[b] for:				
		Pieris brassicae (μg/ml)	*Manduca sexta* (ng/cm²)	*Heliothis virescens* (ng/cm²)	*Mamestra brassicae* (ng/cm²)	*Spodoptera littoralis* (ng/cm²)
CryIA(a)	*aizawai* HD-68	0.8	5.2	90	165	>1,350
CryIA(b)	*berliner* 1715	0.7	8.6	10	162	>1,350
CryIA(c)	*kurstaki* HD-73	0.3	5.3	1.6	2,000	>1,350
CryIB	*thuringiensis* 4412	2.8	>625	>625	>1,350	>1,350
CryIC	*entomocidus* HD-110	6.0	>128	>256	22	104
CryID	*aizawai* HD-68	>75	5	>256	>1,350	>1,350

[a] Proteins were purified from recombinant *E. coli* clones or from *B. thuringiensis* crystal containing a single crystal protein [CryIA(c) from *B. thuringiensis* subsp. *kurstaki* HD-73 and CryIB from *B. thuringiensis* subsp. *thuringiensis* 4412] and solubilized prior to the bioassay.

[b] Data are from reference 42 (for CryIA and CryIB) or H. Höfte, unpublished observations (for CryIC and CryID). LC_{50}, 50% lethal concentration.

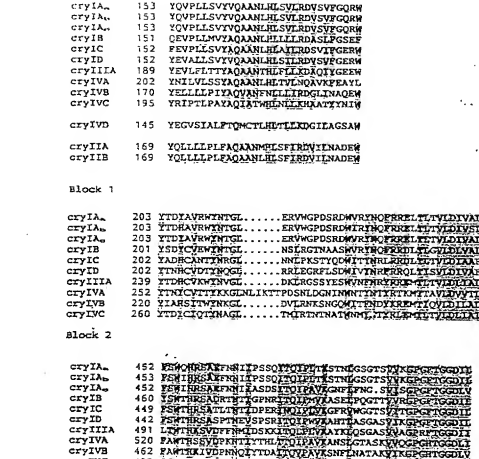

FIG. 2. Conserved amino acid sequence blocks. Amino acid sequence blocks conserved for all crystal proteins encoded by *cry* genes, except CryII and CryIVD, are shown. The position of these blocks in the sequence is shown in Fig. 1. Amino acids marked by the shaded area are identical or conservatively changed for at least 8 of the 10 *cry* sequences. The region in the CryIIA, CryIIB, and CryIVD amino acid sequence homologous to the block 1 sequence is also shown.

proteases which remove 57 N-terminal amino acids (69). As discussed below, *cryIIIA* is homologous to the toxin-encoding domain of the *cryI* and *cryIV* genes and lacks a region corresponding to the 3' half of these molecules. Deletion analysis demonstrated that the gene cannot be truncated at the 3' end without the loss of toxic activity (D. A. Fischhoff, Soc. Invertebr. Pathol. Abstr. Twenty-First Meeting, abstr. 60, p. 60, 1988). Interestingly, the 12 C-terminal amino acids of this protein are highly similar to the sequence at the C terminus of the smallest toxic fragment of CryIA(b), as determined by deletion analysis (described above). In addition, all but three other crystal proteins contain a block of 12 conserved amino acids at a comparable position (Fig. 2, block 5). This makes it reasonable to assume that the C terminus of the toxic fragment of these crystal proteins will also be defined by this sequence. Deletion studies on *cryIA(a)*, *cryIA(c)* (cited above), and *cryIVB* (74) at least do not contradict this assumption.

Diptera-Active Crystal Protein Genes (*cryIV* and *cytA*)

The *cryIV* class of crystal protein genes is composed of a rather heterogeneous group of Diptera-specific crystal pro-

tein genes. Four described genes, as well as the *cytA* gene, were all isolated from the same 72-MDa plasmid present in strains of *B. thuringiensis* subsp. *israelensis*. The *cryIVA*, *cryIVB*, *cryIVC*, and *cryIVD* genes encode proteins with predicted molecular masses of 135, 128, 78, and 72 kDa, respectively. These proteins assemble, together with the 27-kDa *cytA* gene product, in ovoid crystal complexes. A crystal complex with the same or a very similar protein composition has also been observed in one other strain, *B. thuringiensis* subsp. *morrisoni* PG-14. The biochemical properties and the toxicity of the different crystal components have been extensively reviewed by Federici et al. (22). Here we will summarize only the most recent data.

Toxicity tests with crystal protein preparations, derived either from *B. thuringiensis* subsp. *israelensis* crystals (12) or from recombinant *E. coli* (6, 13, 15) or *Bacillus* (16, 25, 106) strains, provide the following picture. All crystal components are, to various extents, toxic against larvae of certain mosquito species. Solubilization of the proteins reduces the toxicity dramatically (50- to 100-fold). This is attributed not to a real loss of toxicity but rather to reduced levels of toxin ingestion by larvae owing to their filter-feeding behavior. No single crystal component is as toxic as the intact crystal complex. One possible explanation for this is that two or more proteins act synergistically, yielding a higher activity than would be expected on the basis of the specific toxicity of the individual proteins. The following lines of evidence suggest that this is indeed the case. The 27-kDa *cytA*-specified protein exhibits no or a rather low dipteran activity (see below). However, subtoxic doses of this protein were reported to significantly increase the toxicity of the ca. 130-kDa (CryIVA and CryIVB) and the ca. 70-kDa (CryIVD) protein fractions of *B. thuringiensis* subsp. *israelensis* crystals against *A. aegypti* larvae (102). Similar observations were described for combinations of CryIVB and CryIVC against larvae of *Culex pipiens* (15).

The architecture of *cryIVA* and *cryIVB* is similar to that of the *cryI* genes. They also encode ca. 130-kDa proteins, which are proteolytically converted into smaller toxic fragments. There is some controversy about the exact molecular masses of the toxic core fragments, which vary from 53 (12) to 78 (13) kDa in different studies. The 3′ halves of these genes are almost identical to each other and are highly similar to the 3′ halves of the *cryI* genes (Fig. 1). This suggests that the toxic fragment of CryIVA and CryIVB is also localized in the N-terminal half. This was confirmed for the *cryIVB* gene product through deletion analysis (13, 15, 74).

The *cryIVC* gene encodes a protein with a predicted molecular mass of 78 kDa. A second open reading frame (ORF2) is localized 45 base pairs downstream from the stop codon of this gene (ORF1). ORF1 shows homology to the 5′ half of the other *cry* genes, whereas ORF2 corresponds to the remaining 3′ part (88). This gene configuration has probably evolved through the insertion of a DNA fragment into a gene that otherwise would encode a ca. 130-kDa polypeptide. When introduced into *B. subtilis* or into a cured *B. thuringiensis* subsp. *israelensis* strain, *cryIVC* directs the expression of a toxic protein of ca. 58 kDa, presumably a proteolytic fragment of the ORF1 gene product. Minor amounts of this 58-kDa protein have also been found in *B. thuringiensis* subsp. *israelensis* crystals (25). The region specifying the active toxin in *cryIVA*, *cryIVB*, and *cryIVC* is highly divergent. Conserved amino acids are restricted mainly to five sequence blocks, which are also conserved for the CryI and CryIIIA proteins (see below).

The *cryIVD* gene encodes a 72-kDa protein (16), which is a major component of the *B. thuringiensis* subsp. *israelensis* crystals (22). This crystal protein, unlike all other known *cry*-encoded proteins, is proteolytically converted into an active fragment of ca. 30 kDa (12, 44, 75). The exact localization of this fragment in the intact protein is not known. Sequence comparisons reveal a rather limited homology to the other crystal proteins in a short region of the molecule (between codons 45 and 174).

The 27-kDa protein encoded by *cytA* shows no sequence homology to the other crystal protein genes. In addition, this protein, purified from *B. thuringiensis* subsp. *israelensis* crystals or from a recombinant *B. subtilis* clone, shows unique functional features. It is cytolytic for a variety of invertebrate and vertebrate cells, including mammalian erythrocytes (87, 95); however, its in vivo insecticidal activity is uncertain. Some authors (68, 95) have shown that this protein has a weak toxicity to *A. aegypti* larvae (50% lethal concentration, 110 to 125 μg/ml, compared with ca. 1 ng/ml for intact crystals), whereas others have failed to demonstrate any insect toxicity (22).

EXPRESSION OF *cry* GENES

The structure of the *cryIA(a)* promoter region has been reviewed previously (97). In brief, in *B. thuringiensis*, *cryIA(a)* is transcribed from two start sites, located ca. 16 base pairs apart: Bt I, which is activated early in sporulation (t_1 to t_2 [where t_n indicates the number of hours after the onset of sporulation]), and Bt II, which is activated at midsporulation (t_4 to t_5 (101). In vitro transcription from Bt I is catalyzed by a specific *B. thuringiensis* ribonucleic acid (RNA) polymerase containing a new sigma subunit of ca. 35 kDa (83); vegetative genes are transcribed by the predominant RNA polymerase, which contains a sigma subunit of 61 kDa. Transcription from Bt II requires a second RNA polymerase containing another new sigma subunit of ca. 28 kDa (K. L. Brown and H. R. Whiteley, unpublished observations). The *cryIA(b)* and *cryIA(c)* genes are reported to have the same promoter structure as *cryIA(a)* (2, 88), and transcription of several other genes (*cryIB*, *cryIIA*, and *cytA*) requires the sigma-35-containing RNA polymerase (Brown and Whiteley, unpublished). Lastly, many crystal protein genes have a strong terminator. Wong and Chang (100) showed that the presence of the terminator significantly enhances the stability of crystal protein messenger RNA (mRNA).

In Spo⁺ *Bacillus subtilis*, transcription of *cryIA(a)* is predominantly from the Bt I start site (H. E. Schnepf, W. R. Widner, K. L. Brown, and H. R. Whiteley, unpublished observations), and preliminary evidence (Brown and Whiteley, unpublished) indicates that in vitro transcription requires a *B. subtilis* RNA polymerase containing a sigma subunit of ca. 35 kDa. The identity of the RNA polymerase responsible for the much weaker transcription from Bt II in Spo⁺ cells has not been investigated. Transcription mapping showed that Bt I is utilized in the *spoIIC*, *spoIIAC*, and *spoIIIE* sporulation mutants of *B. subtilis*, but not in the *spoIIG41* mutant. Assays of chloramphenicol acetyltransferase fused to the *cryIA(a)* promoter showed little or no utilization of the promoter (<5% activity) in *spo0A*, *spo0B*, *spo0F*, *spo0H*, *spoIIE*, and *spoIIAA* mutants and partial activity (40%) in *spo0J* mutants. However, the dependence on sporulation may be related to the vector used in cloning, since Shivakumar et al. (86) reported that a crystal protein gene cloned into a different plasmid was not regulated by sporulation.

In *E. coli*, *cryIA(a)* is transcribed from a site at or very near Bt II; the identity of the polymerase responsible for this transcription has not been established. Unexpectedly, expression of the *cryIA(a)* gene in *E. coli*, but not in *B. subtilis*, is regulated negatively by a region of DNA located at about position −87 to −258 relative to Bt I. Deletion or interruption of this region enhances gene expression approximately 10-fold (80). Deletion of this region also yields increased expression of *cryIA(b)* (88). The biochemical basis for this regulatory mechanism has not been investigated.

There is one additional regulatory mechanism that should be mentioned. Full expression of the *cytA* gene in *E. coli* (i.e., abundant synthesis of CytA, toxicity to mosquito larvae, and haemolytic activity) requires the presence of a segment of *B. thuringiensis* subsp. *israelensis* DNA located about 4 kilobases upstream from the *cytA* gene (68). This region is not required for expression of the *cytA* gene in *B. subtilis* (95). The upstream DNA that is involved in full expression encodes a 20-kDa peptide (1), which acts in *trans* at the posttranscriptional level to increase the amount of CytA produced. This effect occurs after the initiation of translation; possibly the 20-kDa peptide is involved in protecting CytA from degradation. The gene encoding the 20-kDa peptide is apparently transcribed as part of an operon which contains *cryIVD*, and its product is synthesized concurrently with CytA, beginning at about t_2. Interestingly, the 20-kDa protein is present in *B. thuringiensis* subsp. *israelensis* crystals; thus, crystals may contain not only mixtures of several insecticidal proteins, but also regulatory proteins or proteins such as the *orf2* product (part of the *cryIIA* operon) which may have some structural function.

MODE OF ACTION

Most studies of the histopathology and mode of action have been carried out on lepidopteran larvae with often rather ill-defined toxin preparations derived from whole *B. thuringiensis* crystals. In summary, these investigations (reviewed in detail in reference 63) showed that the crystal proteins dissolve in the larval insect midgut and are proteolytically converted into a toxic core fragment. The epithelial cells of the midgut, or cultured insect cells, rapidly swell and burst after toxin treatment. The absence of a lag period before the appearance of the first symptoms suggests that the toxin does not need to be internalized to mediate cytotoxicity. Recent investigations shed more light on the mechanism of action. Knowles and Ellar (49) studied the effect of *B. thuringiensis* toxins on the permeability of cultured insect cells by using radioactively labeled small molecules. After toxin treatment, they observed a rapid release of the small molecules, which leaked out of the cell before the larger ones. In addition, cytolysis was inhibited or delayed by osmotic protectants. On the basis of these and other observations, the authors proposed that all four of the *B. thuringiensis* toxin preparations they tested (derived from *B. thuringiensis* subspp. *kurstaki* HD-1, *aizawai* HD-249, *thuringiensis* HD-50, and *israelensis* IPS-73) provoke a colloid-osmotic lysis as described for mellitin. According to this model, these toxins induce the formation of small, nonspecific pores (0.5 to 1 nm) in the membrane of susceptible cells, resulting in a net influx of ions and an accompanying inflow of water. As a result, the cells swell and lyse.

One has to remark here that results from investigations on cultured insect cells should always be confirmed by studies on larval gut tissue for the following reasons: (i) the available cell lines are derived from tissues other than the midgut and are therefore not a primary target of *B. thuringiensis* toxins in vivo; (ii) toxic effects on insect cell lines are observed only at much higher toxin concentrations (100-fold) than those required for in vivo toxicity; and (iii) the specificity for cell lines derived from different insect species does not strictly correlate with the in vivo host range of crystal proteins (48, 99).

Biochemical studies on isolated midgut membranes lead to slightly different conclusions. Sacchi et al. (76) studied the K^+-amino acid cotransport into brush border membrane vesicles (BBMVs) prepared from midguts of *Pieris brassicae* larvae. Treatment with the toxin (i.e., purified, solubilized, and activated crystals from *B. thuringiensis* subsp. *kurstaki* HD-1 and *B. thuringiensis* subsp. *thuringiensis* 4412) immediately inhibited the transport of labeled histidine driven by a K^+ gradient. As they observed no influence of the toxin on the K^+-amino acid cotransporter itself, they reasoned that the K^+ gradient must be dissipated through formation of channels in the brush border membrane upon toxin treatment. The amino acid transport in this tissue can also be driven by a Na^+ gradient, although less efficiently. However, the authors failed to demonstrate any influence of the toxin on the Na^+-driven amino acid cotransport. From this, they concluded that the channels might be K^+ specific.

In conclusion, these studies suggest that *B. thuringiensis* toxins induce the formation of pores in the membrane of both midgut epithelial cells and cultured insect cells. Direct permeability measurements on BBMVs with radioactively labeled ions are needed to sort out whether the initially formed pores are K^+ specific. In relation to this, it will be interesting to see whether the toxin induces pore formation indirectly through interaction with resident membrane proteins or directly through insertion into the membrane.

The mechanism of the cytotoxic activity has been addressed in several other studies summarized in the discussion section of reference 49. A rather unusual observation was made by English and Cantley (21), who demonstrated that toxin preparations from *B. thuringiensis* subsp. *kurstaki* crystals nonspecifically inhibited a K^+-adenosine triphosphatase from various sources (insect cells, human erythrocytes, and dog kidneys). However, the inhibition occurred only at very high toxin concentrations (100 μg/ml), and it seems unlikely that it plays a significant role in insect toxicity.

Factors That Determine Specificity

In the previous sections, we presented an overview of the variation in insect host range among individual crystal proteins. One of the most intriguing questions relates to the molecular basis for this extreme insect specificity. The most obvious factors that may influence the host range of a crystal protein are (i) differences in the larval gut affecting the solubilization and/or processing efficiency of the protoxin and (ii) the presence of specific toxin-binding sites (receptors) in the gut of different insects. It has been shown that certain insects have a low susceptibility for *B. thuringiensis* crystal proteins owing to the inefficient solubilization of the crystals. In vitro, solubilization of crystals significantly enhances the toxic activity (45, 59). However, dramatic differences in specificity are maintained after solubilization of the crystal proteins (Table 3).

Most experiments in which the toxic activities of protoxin and activated toxin were compared suggest that susceptibility for certain crystal proteins is independent of the activation (45, 63). However, for one crystal protein, evidence was

provided that a protoxin can be activated into either a dipteran or a lepidopteran toxin, depending on the source of the proteolytic enzymes (31). In brief, Haider et al. (29, 31) cloned a gene coding for a 130-kDa protein from *B. thuringiensis* subsp. *aizawai* into *E. coli*. Treatment of the 130-kDa protein with trypsin yielded a 55-kDa peptide that was toxic to cultured lepidopteran cells (*Choristoneura fumiferana*) but not to cultured dipteran cells. When the trypsin-activated toxin was exposed to mosquito gut proteases, a 53-kDa peptide was detected, and the preparation was toxic only to cultured mosquito cells. Binding studies showed that the trypsin-activated 55-kDa peptide bound to a 68-kDa protein present in the membranes of cultured lepidopteran cells. A receptor for the Diptera-specific 53-kDa peptide has not been identified.

Recent experiments (37, 38) suggest that the interaction with high-affinity binding sites on the insect midgut epithelium may, to a large extent, determine the host spectrum of *B. thuringiensis* crystal proteins. In a first set of experiments, binding studies were carried out with two ^{125}I-labeled toxins [CryIA(b) and CryIB] on BBMVs prepared from the larval midgut of *M. sexta* and *P. brassicae*, respectively. The CryIA(b) toxin is active against both insects, whereas the CryIB toxin acts only against *P. brassicae* (Table 5). Interestingly, the activated CryIA(b) toxin binds with high affinity to BBMVs derived from both insects, whereas the CryIB toxin shows saturable binding only to *P. brassicae* vesicles. Heterologous competition experiments with other toxins provide further evidence for a correlation between toxicity and specific binding. CryIA(a) and CryIA(c), which are equally toxic for *M. sexta* (Table 5), competed with the labeled CryIA(b) toxin for the same binding site, whereas the dipteran-specific CryIVB and the coleopteran-specific CryIIIA did not compete. Unexpectedly, competition experiments with labeled and unlabeled CryIA(b) and CryIB toxins showed that they recognize distinct binding sites on the same BBMVs from *P. brassicae*.

Very little is known so far about the biochemical nature of these specific toxin-binding sites. Binding studies on BBMVs after treatment with various proteases suggest that the binding sites consist at least of a proteinaceous component (37; H. van Mellaert, personal communication). Knowles and Ellar (48) identified a 146-kDa membrane glycoprotein from *Choristoneura fumiferana* cells as a possible toxin receptor. Knowles et al. also reported that *N*-acetylgalactosamine protected these cells against *B. thuringiensis* subsp. *kurstaki* HD-1 preparations, suggesting that this sugar is a part of the binding site (51). However, *N*-acetylgalactosamine did not interfere with the specific binding of ^{125}I-labeled CryIB toxin to *P. brassicae* BBMVs or with the binding of ^{125}I-labeled CryIA(b) toxin to *M. sexta* vesicles (van Mellaert, personal communication). These results also suggest that the effect of *B. thuringiensis* toxins on cultured cell lines is not directly comparable to the in vivo activity.

Conserved Features of *cry* Genes

The *cry* genes described so far share the following characteristics. They encode insecticidal proteins, either of 130 to 140 kDa or of ca. 70 kDa, containing a toxic fragment of 60 ± 10 kDa. One exception is the *cryIVD* protein, which contains a ca. 30-kDa active core component (12). For the 130- to 140-kDa proteins, the toxic segment is localized in the N-terminal half of the protoxin. The C-terminal part of the ca. 130-kDa proteins (CryI, CryIVA, and CryIVB) is not essential for toxicity, but is the most highly conserved domain of these crystal proteins (Fig. 1).

In the amino acid sequence corresponding to the toxic domain, five highly conserved sequence blocks can be distinguished in all but three *cry*-specified proteins (Fig. 1 and 2). Within these conserved sequence blocks, no (or relatively few) gaps were needed for the alignment of the identical or related amino acids. These blocks are separated by highly variable sequences of various lengths for the different crystal proteins. Exceptions are the CryII proteins and CryIVD, which show significant homology only to the other *cry* proteins in a region corresponding to block 1. Another recurring feature for all crystal proteins except CryII and CryIVD is the presence of a stretch of hydrophobic amino acids at a comparable position within the 120 *N*-terminal amino acids. This amino acid stretch shows the properties of a predicted transmembrane sequence according to Eisenberg et al. (20) (Fig. 1). Remarkably, within this region only the hydrophobic character and not the identity of the amino acids is conserved, strongly supporting a functional significance. It has been proposed that the conserved hydrophobic region plays a role in an interaction of the toxin with the membrane of midgut epithelial cells (79), but direct experimental evidence for such interactions is lacking.

USE OF *B. THURINGIENSIS* CRYSTAL PROTEINS IN PLANT PROTECTION

B. thuringiensis has proven to be a valuable alternative to conventional insecticides. It is highly active and harmless to the environment owing to its specificity. Formulations of *B. thuringiensis* spore-crystal mixtures are commercially available for use as biological insecticides in agriculture and forestry. *B. thuringiensis* subsp. *israelensis*, active against larvae of mosquitoes and blackflies, is being used to control vectors of a variety of human and animal diseases. An important disadvantage is the rather restricted host range of the existing *B. thuringiensis* sprays. Screening samples from different environments may yield *B. thuringiensis* strains with broader host ranges or new specificities. The host range of strains used commercially can be expanded through the introduction of new crystal protein genes. This can be achieved through conjugation (reviewed in reference 11) of plasmids from other *B. thuringiensis* strains or through direct transformation of crystal protein genes cloned in a *B. thuringiensis* replicon (34). In this respect, the recent development of an efficient transformation system involving the use of electroporation (J. Mahillon, personal communication) will greatly facilitate future manipulation of *B. thuringiensis* strains.

Another problem related to commercial *B. thuringiensis* preparations is the limited field stability. Here, the introduction of crystal protein genes into plant-associated microorganisms might provide a valuable alternative. One proposal is to introduce these genes into endophytic bacteria (14). Another approach is the Tn*5*-mediated insertion of a *cryIA(b)* gene into the chromosomes of *Pseudomonas fluorescens* and *Agrobacterium radiobacter* strains that colonize corn roots (72). Transformants of the *P. fluorescens* and *A. radiobacter* strains expressed the crystal protein and were toxic against *M. sexta*.

Transgenic Plants

Recently, the feasibility of generating insect-resistant transgenic crops by using *B. thuringiensis* crystal proteins

was demonstrated. In all previously described experiments (5, 23, 90), an *Agrobacterium tumefaciens*-based transformation system (28) was used. Modified crystal protein genes were placed under the control of a promoter and a 3' end of a plant gene. Vaeck et al. (90) used derivatives of a *cryIA(b)* gene under control of the mannopine synthase TR2' promoter from the octopine Ti plasmid of *A. tumefaciens*. Three groups of toxin gene cassettes were used: the intact gene; 3' deletion derivatives containing the toxin-encoding half of the gene; and fusions between the toxin-encoding part of the gene and *neo*, a selectable marker gene derived from Tn5 which confers kanamycin resistance (neomycin phosphotransferase activity) to transformed plant cells. These fusion genes expressed chimeric proteins with both insecticidal and neomycin phosphotransferase activities. This allowed the selection of plant transformants with high insecticidal activity through selection on high doses of kanamycin. Transformed tobacco plants toxic against *M. sexta* larvae were obtained, and the amount of crystal protein detected immunologically in the plant tissue correlated well with the level of insect resistance. It is remarkable that levels of crystal protein that killed insects were obtained only when the truncated or fusion genes were used. It is not known why the intact gene is not expressed in the plant cells. Transgenic tobacco plants tested in field trials were fully protected against *M. sexta* and *H. virescens* damage (G. Warren, personal communication). Fischhoff et al. (23) used a similar approach with 3'-deleted derivatives of a *cryIA(b)* gene under the control of the 35S promoter of cauliflower mosaic virus. They transformed tomato plants and obtained plants that were toxic for *M. sexta*. Barton et al. (5) used a 3'-deleted *cryA(a)* gene under the control of the 35S promoter. They also inserted the 5' untranslated leader of the coat protein gene (gene 4) of alfalfa mosaic virus upstream of the toxin gene. This leader might enhance the efficiency of translation initiation. Tobacco plants resistant against *M. sexta* larvae were obtained.

Similar approaches are now being used for other commercial crops with important lepidopteran or coleopteran pests and for which a transformation system is available (potato, cotton, etc.). A recent important development is the use of high-velocity microprojectiles (47, 65), making it possible to transform other crops, especially monocots (e.g., corn), which cannot be transformed by the *Agrobacterium* system.

Resistance Development

Despite the wide use of *B. thuringiensis* formulations over the last 20 years, no cases of resistance development in the field have been reported. An important factor that may have contributed to this is the low persistence of *B. thuringiensis* in the environment. The situation might change dramatically when the use of insect-resistant transgenic plants becomes widespread, since several generations of insects per year will be continuously exposed to crystal proteins, providing an ideal environment for the development of resistance. In this respect, it is interesting to mention the only reported case of resistance development in the laboratory.

McGaughey (66) demonstrated that the Indian mealmoth (*Plodia interpunctella*), a lepidopteran pest of stored grain and grain products, can develop resistance against Dipel (Abbot Laboratories), a commercial spore-crystal formulation of *B. thuringiensis* subsp. *kurstaki* HD-1. A colony of *Plodia interpunctella* was reared on a diet containing Dipel at a dose expected to produce 70 to 90% larval mortality. In two generations, resistance increased ca. 30-fold, and after 15 generations, a plateau 100 times higher than the control level was reached. The resistance remained stable in the absence of selective pressure and was inherited as a recessive trait. An interesting observation was that other *B. thuringiensis* strains were still highly active against the resistant insects (67). An attractive hypothesis is that the selected *Plodia interpunctella* strain is resistant only to CryIA toxins present in the Dipel preparation, but not to other types of toxins. If this is so, then the simultaneous expression in transgenic plants of two or more toxins that act independently against the same insect (perhaps through the recognition of distinct binding sites) should reduce the probability that resistant insect populations will develop. Overall, the study of the molecular basis of resistance against selected toxins deserves intensive study and may provide important clues for the understanding of the basis of the insect specificity.

CONCLUSIONS AND PERSPECTIVES

It has become clear in recent years that *B. thuringiensis* is provided with a surprisingly large and variable family of insecticidal proteins. In this review, we have presented a classification of crystal protein genes based on insect specificity and the primary structure of the proteins. Knowles and Ellar (50) have also pointed out that crystal proteins can be grouped according to host range. They proposed five pathotypes: four of these correspond to the four classes shown in Table 1, and the fifth contains nontoxic proteins. We have not included the fifth class, because the structures of nontoxic crystal proteins have not been determined. To date, 14 gene types can be distinguished in our classification. We have assigned these genes to the minimum number of categories: the 13 related *cry* genes have been placed in four classes according to structure and host range. A totally unrelated gene (*cytA*) has been placed in a separate, fifth category. Additional gene types will undoubtedly be discovered in the current intensive screening efforts to isolate crystal proteins with different host ranges. We hope that Table 1 will provide a useful framework for the classification of these additional genes.

Progress has been made in the understanding of the biochemical mechanism of toxicity and the factors that determine the extreme specificity. Data from in vitro experiments strongly suggest that activated toxins induce the formation of pores in the membrane of susceptible cells and that they recognize high-affinity binding sites (putative receptors) on the midgut epithelium of susceptible insects only. Several interesting questions relating to the structure-activity relationship of *B. thuringiensis* toxins have not yet been answered. It is not known whether the active toxin, like several other protein toxins (70), consists of two or more domains that mediate different steps in the toxic action (e.g., receptor binding, pore formation). The comparison of the deduced amino acid sequences revealed a number of sequence elements conserved for most crystal proteins; however, it is not known whether these conserved elements also have any functional role in the toxic activity. In vitro mutagenesis techniques (94) and/or the functional in vitro assays for receptor binding (37, 38) and membrane permeability (76) will shed more light on these matters. Also, monoclonal antibodies can be used to map functional domains on the toxin molecule. Finally, two recent reports describe the in vitro crystallization of CryIIIA and some preliminary X-ray diffraction data (26, 62). This approach will provide the necessary structural data for a rational study of the molecular basis of the insecticidal activity.

The discovery of putative receptor sites for *B. thuringiensis* toxins provides promising avenues for future research. Binding studies involving the use of different toxins and a variety of insect species will show whether toxicity is always associated with high-affinity binding to midgut membranes. Interesting results can also be expected from the isolation and characterization of the putative receptor molecules. Do these receptors have any physiological role in healthy insects, and can one identify related molecules in the midgut epithelium of nonsusceptible insects? Answers to these questions might provide strategies for the development of new generations of insecticides.

New insights on the regulation of crystal protein gene expression were provided by the identification of two sporulation-specific sigma factors, the finding of negative regulation of crystal protein gene expression in *E. coli*, and the description of a protein affecting the expression of the CytA gene at a posttranslational level in *E. coli*. The recent development of transformation systems for *B. thuringiensis* will facilitate the study of crystal protein gene regulation in its original host. The results from these studies are essential for the development of new *B. thuringiensis* strains expressing combinations of crystal proteins with interesting insecticidal spectra.

The expression of dipteran-active crystal proteins in other microorganisms, such as *B. sphaericus* or cyanobacteria (W. Chungjatupornchai, personal communication), may allow more effective control of medically important dipteran insect vectors. Finally, it may be possible to produce a number of agriculturally important crops from transgenic plants that express *B. thuringiensis* crystal proteins. This will provide a potentially powerful alternative to chemical insecticides in agriculture.

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